REMARKS

After entry of this amendment, claims 1-35, 38-44 and 47-66 are pending, of which claims 7-9, 15-35, 38-44 and 47-63 are withdrawn. Claims 36, 45 and 46 have been cancelled without prejudice or disclaimer. New claims 64-66 have been added and find support *inter alia* in the original claims. New claim 64 finds further support in the specification, for example, at page 42, lines 4-6. New claim 65 finds further support in the specification, for example, at page 12, lines 30-31, and page 26, lines 17-18. New claim 66 finds further support in the specification, for example, at page 39, lines 11-22. The claims have been amended without prejudice or disclaimer and find support *inter alia* in the original claims. Claim 1 finds further support in the specification, for example, at page 42, lines 2-3. Withdrawn claim 47 has been amended to depend from examined claim 1 and to incorporate the subject matter recited in cancelled claim 46. Support is found *inter alia* in the original claims. No new matter has been added.

In view of the present amendment to withdrawn claim 47, rejoinder of claim 47 is respectfully requested.

Claim Objection

Claim 6 is objected to for reciting non-elected sequences, namely SEQ ID NO: 53, encoding SEQ ID NO: 54, and SEQ ID NO: 113, encoding SEQ ID NO: 114. Applicants respectfully disagree.

It is noted initially that SEQ ID NO: 53 and 113 both encode a polypeptide with Δ5-elongase activity as provided in the specification, for example, at pages 17-18. As confirmed by the Examiner in the Office Action at page 2, the election to the sequences that correspond to the elected subject matter is for search purposes only, i.e. species election. Accordingly, upon allowance of the generic claim or the claims directed to the elected species, Applicants respectfully request rejoinder of the non-elected species. 37 CFR § 1.141; MPEP § 809.02(a).

Specification

The specification is objected to as being unclear as to whether it contains a brief description of the drawings. The specification is further objected to for not containing the preferred headings for each section as suggested in the MPEP. In response, the specification has been amended to include the appropriate section headings as suggested in the MPEP.

Additionally, a section entitled "BRIEF DESCRIPTION OF THE DRAWINGS" has been inserted at page 9 to accommodate the figure legends found on top of each drawing. Support is found *inter alia* in the drawings containing Figures 1-33. Further support for the brief description of Figure 23 is found in the specification, for example, at page 143, lines 2-3. Further support for the brief description of Figure 28 is found in the specification, for example, at page 42, line 34, to page 43, line 1. No new matter has been added.

The abstract of the disclosure is objected to for containing more than one paragraph. In response, Applicants submit herewith a new abstract on a separate sheet pursuant to 37 CFR § 1.72. Support for the new abstract is found *inter alia* in the abstract as originally filed. No new matter has been added.

In view of the present amendments, reconsideration and withdrawal of the objections is respectfully requested.

Double Patenting

Claims 1-6 and 10-14 are provisionally rejected for obviousness-type double patenting over claims 1, 2 and 5-11 of co-pending Application No. 10/566,944. Because this is a provisional double patenting rejection, Applicants will consider filing an appropriate terminal disclaimer upon an indication that the claims are allowable.

Claim Rejection – 35 U.S.C. § 102

Claims 1-5 and 10-14 are rejected under 35 U.S.C. § 102(b) as being anticipated by Drexler *et al.* (hereinafter "Drexler").

The Examiner contends that Drexler teaches a process to produce compounds of Formula I in a plant by introducing into the plant coding sequences for a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-elongase, and a Δ4-desaturase. The Examiner further alleges that Drexler teaches transformation of canola with desaturase coding sequences to produce polyunsaturated fatty acid (18:3). The Examiner additionally argues that the recited substituents of R2 and R3, as well as the percentage of Formula I compounds produced, would be inherent in seeds made by the same process. The Examiner also asserts that the specification does not define Δ5-elongase activity. Office Action at page 6. Applicants strongly disagree.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegall Bros., Inc. v. Union Oil Co.*, 814 F.2d 628, 631 (Fed. Cir. 1987). "Rejections under 35 U.S.C. § 102 are proper only when the claimed subject matter is identically disclosed or described in the prior art. Thus, it is not enough that the prior art reference discloses part of the claimed invention, which an ordinary artisan might supplement to make the whole, or that it includes multiple, distinct teachings that the artisan might somehow combine to achieve the claimed invention. The prior art reference must clearly and unequivocally disclose the claimed invention or direct those skilled in the art to the invention without any need for picking, choosing, and combining various disclosures not directly related to each other by the teachings of the cited reference." *Net MoneyIN Inc. v. VeriSign Inc.*, 545 F.3d 1359 (Fed. Cir. 2008) (holding "that unless a reference discloses within the four corners of the document not only all the limitations claimed but also all of the limitations arranged or combined in the same way as recited in the claim, it cannot be said to prove prior invention of the thing claimed and, thus, cannot anticipate under 35 U.S.C. § 102.").

It is noted initially that Δ5-elongase activity as recited in the claims is well defined throughout the specification. For example, the activity of a Δ5-elongase suitable for practicing the claimed process is described in detail in the specification, for example, at page 41, line 34, to page 42, line 8. As described therein, the Δ5-elongase according to the present application preferably converts only unsaturated C_{20}-fatty acids but not C_{22}-fatty acids. The specification further describes that the Δ5-elongase according to the present application converts advantageously only C_{20}-fatty acids with one double bond in the Δ5-position, with ω3-C_{20}-fatty acids being preferred (EPA). Such substrate specificity is further demonstrated in, for example, Figures 24, 25 and 28 using Δ5-elongases isolated from *Ostreococcus tauri*. Thus, contrary to the Examiner's assertion, Δ5-elongase activity is well defined and described throughout the specification as being capable of converting unsaturated fatty acids.

Drexler provides a general review of biosynthesis of arachidonic acid ("ARA"), eicosapentaenoic acid ("EPA"), and docosahexaenoic acid ("DHA"), as summarized in Figure 6 at page 795. However, contrary to the Examiner's assertion, Drexler does not teach production of very long-chain polyunsaturated fatty acids ("VLCPUFA") by introducing into one single plant nucleotide sequences encoding a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-

elongase, and a Δ4-desaturase. Specifically, Drexler does not teach a Δ5-elongase that elongates only unsaturated C_{20}-fatty acids as recited in the present claims. The lack of such teaching is further evidenced from the statement found at page 796 of Drexler, where it states, "[t]he only enzyme which has not yet been identified by explicit functional expression studies of the isolated cDNA is a Δ5-specific elongase limited to the elongation of C_{20}- to C_{22}-fatty acids and that does not initiate the several elongation cycles as typical for a mammalian enzyme." Drexler at page 796, left Col., lines 10-15.

Moreover, Applicants further note that, as described in Drexler at pages 795-796, different types of elongases exist. For example, the two elongations of Δ9-18:1 to Δ13-22:1 as catalyzed by the KCS enzyme and its allies involve exclusively intermediates of the acyl-CoA pool. The elongations in DHA biosynthesis, on the other hand, presumably depend on several rounds of enzyme-catalyzed shuttling of the acyl group between lipid-linked oxygen ester (for desaturation) and CoA-bound thioester forms (for elongation). Recent investigations further show that the PUFA-elongating enzymes are encoded by a gene family completely different from the KCS family. See Drexler at page 795, right Col., citing to Zank *et al.* (Plant J., 2002, 31: 255-268).

The elongase genes disclosed in the references cited in Drexler, such as the KCS-cDNA isolated from *L. douglasii* (citing Cahoon *et al.*, Plant Physiology, 2000, 124: 243-251; hereinafter "Cahoon"; copy attached), although could be considered as a Δ5-elongase, it actually encodes a FAE-type elongase which elongates primarily **saturated** fatty acids (e.g., 16:0 or 18:0) but not unsaturated fatty acids (e.g., 16:1 or 18:1). See Cahoon at page 247, paragraph bridging left and right columns. Contrary to those FAE-type elongases disclosed in Cahoon and Drexler, the elongases according to the present application, the ELO-type Δ5-elongases, elongate **unsaturated** fatty acids with at least one double bond as discussed above.

As discussed above, Drexler acknowledges that the enzyme with a Δ5-specific elongase activity limited to the elongation of C_{20}- to C_{22}-fatty acids has not yet been identified. Thus, it is clear that an ELO-type Δ5-elongase that elongates only unsaturated C_{20}-fatty acids was not known in the art and was disclosed for the first time in the present application.

Without acquiescing to the merits of the Examiner's characterization of Drexler and arguments, and merely for further clarifying and differentiating the claimed subject matter from the cited reference, claim 1 has been amended without prejudice or disclaimer to specify that the

recited Δ5-elongase elongates only unsaturated C_{20}-fatty acids. As discussed above, Drexler, or the references cited therein, does not teach a Δ5-elongase that elongates unsaturated C_{20}-fatty acids. Because Drexler does not teach each and every limitation as set forth in the present claims, either expressly or inherently, within its four corners, Drexler does not anticipate the claims as amended.

For at least the above reasons, Applicants submit that Drexler does not anticipate the claimed subject matter as amended. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

Claims 1-5 and 10-14 are rejected under 35 U.S.C. § 102(a) or 102(e) as anticipated by Kinney *et al.* (hereinafter "Kinney").

The Examiner asserts that Kinney teaches a process of producing compounds of Formula I by introducing into a soybean embryo coding sequences for a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-elongase, and a Δ4-desaturase. The Examiner further alleges that Kinney discloses transformed somatic embryos from soybean comprise about 1-3% DHA and greater than 20% of PUFA (18:3). The Examiner additionally contends that the percent by weight of compounds of Formula I recited in the present claims would be inherent in seeds produced by the process taught in Kinney. Office Action at pages 7-8. Applicants strongly disagree.

As acknowledged by the Examiner, Kinney discloses production of fatty acids in somatic embryos of soybean that were transformed with coding sequences for a Δ6-desaturase, a Δ5-desaturase, a Δ17-desaturase, a Δ4-desaturase, an elongase isolated from *M. alpina*, and an elongase isolated from *Pavlova* (Example 13). Fatty acid compositions produced in the transformed somatic embryos were analyzed and provided in, for example, Tables 9 and 10. Although procedures for embryo desiccation and germination to obtain mature seeds are described in Example 9, no such seeds were produced from those transgenic somatic embryos. Nor are fatty acid compositions of transgenic seeds expressing a Δ6-desaturase, a Δ5-desaturase, a Δ17-desaturase, a Δ4-desaturase, an elongase isolated from *M. alpina*, and an elongase isolated from *Pavlova* provided. Since somatic embryos are totally different from seeds and no seeds were produced from the somatic embryos transformed with coding sequences for the recited desaturases and elongases, Kinney does not teach each and every limitation as set forth in the present claims, either expressly or inherently, within its four corners, and thus, does not anticipate the claims as amended.

For at least the above reasons, Applicants submit that Kinney does not anticipate the claimed subject matter as amended. Reconsideration and withdrawal of the rejection is thus respectfully requested.

Claim Rejections – 35 U.S.C. § 103

Claims 1-6 and 10-14 are rejected under 35 U.S.C. § 103(a) as being obvious over Drexler in view of Geneseq Accession No. ABV74261.

The Examiner's reliance on Drexler is the same as discussed above in the anticipation rejection. Specifically, the Examiner asserts that Drexler teaches a process to produce compounds of Formula I in a plant by introducing into the plant coding sequences for a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-elongase, and a Δ4-desaturase. The Examiner acknowledges that Drexler does not teach the Δ6-elongase of SEQ ID NO: 28 encoded by SEQ ID NO: 27, but relied on ABV74261 for such teaching. Based on the above assertions, the Examiner contends that one skilled in the art would have been motivated to substitute the enzyme coding sequences used in Drexler's method with other known sequences such as that of ABV74261. The Examiner further asserts that the particular R2 and R3 constituents and levels of PUFAs would be the optimization of process parameters. The Examiner thus concludes that the claimed process would have been obvious absent evidence to the contrary. Applicants respectfully disagree.

The Examiner bears the initial burden of establishing *prima facie* obviousness. *See In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). To support a *prima facie* conclusion of obviousness, the prior art must disclose or suggest all t he limitations of the claimed invention. *See In re Lowry*, 32 F.3d 1579, 1582, 32 USPQ2d 1031, 1034 (Fed. Cir. 1994).

The discussion above concerning Drexler is equally applicable here and thus, is incorporated by reference in its entirety. As discussed above, Drexler does not teach production of VLCPUFA by introducing into one single plant nucleotide sequences encoding a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-elongase, and a Δ4-desaturase. Particularly, as noted by Drexler, Δ5-specific elongase capable of elongating only unsaturated C_{20}-fatty acids has not yet identified at the time. Moreover, a fair reading of Drexler indicates that Drexler provides only an overview of existing strategies and projects used in the production of fatty acids in plants,

including the problems encountered by those strategies and projects, but not any solution to those problems. Thus, Drexler does not teach one skilled in the art to practice the process for producing compounds of Formula I in a plant as alleged by the Examiner.

The combination of Drexler with ABV74261 does not remedy this deficiency. As noted by the Examiner, ABV74261 discloses a Δ6-elongase coding sequence. Drexler and ABV74261, even if combined, still do not teach or suggest a Δ5-specific elongase capable of elongating only unsaturated C_{20}-fatty acids coding sequence, which, according to Drexler, has not yet been identified. Moreover, it is noted that ABV74261 provides only the sequence information of a Δ6-elongase but not any solution to the problems encountered by the prior art strategies and projects as summarized in Drexler. Thus, even if combined, Drexler and ABV74261 still do not teach a skilled artisan to practice the process that is allegedly taught in Drexler.

Because Drexler and ABV74261, alone or in combination, do not teach or suggest all the limitations of the claimed process, a *prima facie* case of obviousness has not been established. For at least the above reasons and in view of the present amendments, reconsideration and withdrawal of the rejection is respectfully requested.

Claims 1-6 and 10-14 are further rejected under 35 U.S.C. § 103(a) as being obvious over Kinney in view of Geneseq Accession No. ABV74261.

The Examiner's reliance on Kinney is the same as discussed above in the anticipation rejection. Specifically, the Examiner asserts that Kinney teaches a process to produce compounds of Formula I by introducing into a soybean embryo coding sequences for a Δ6-elongase, a Δ6-desaturase, a Δ5-desaturase, a Δ5-elongase, and a Δ4-desaturase. The Examiner further alleges that the claimed fatty acid compositions would be inherent in seeds produced by such a method. Applicants respectfully disagree.

The discussion above concerning Kinney is equally applicable here and thus, is incorporated by reference in its entirety. As discussed above, Kinney does not teach production of seeds transformed with coding sequences for the recited desaturases and elongases. Nor does Kinney teach fatty acid compositions of such transgenic seeds. The combination of Kinney with ABV74261 does not remedy this deficiency. As noted above, ABV74261 provides only the sequence information of a Δ6-elongase. Thus, even if combined, Kinney and ABV74261 still do

not teach a skilled artisan transgenic seeds expressing the recited desaturases and elongases or a process of producing compounds in such transgenic seeds.

Because Kinney and ABV74261, alone or in combination, do not teach or suggest all the limitations of the claimed process, a *prima facie* case of obviousness has not been established. For at least the above reasons and in view of the present amendments, reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

For at least the above reasons, Applicants respectfully request withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Applicants reserve all rights to pursue the non-elected claims and subject matter in one or more divisional applications, if necessary.

Accompanying this response is a petition for a one-month extension of time to respond to the Office Action mailed April 30, 2010 with the required fee authorization. No further fee is believed due. However, if any additional fee is due, the Director is hereby authorized to charge our Deposit Account No. 03-2775, under Order No. 13987-00020-US from which the undersigned is authorized to draw.

Respectfully submitted,

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Attachment: Cahoon *et al.*, Plant Physiology, 2000, 124: 243-251

Production of Fatty Acid Components of Meadowfoam Oil in Somatic Soybean Embryos

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The seed oil of meadowfoam (*Limnanthes alba*) and other *Limnanthes* spp. is enriched in the unusual fatty acid Δ^5-eicosenoic acid ($20{:}1\Delta^5$). This fatty acid has physical and chemical properties that make the seed oil of these plants useful for a number of industrial applications. An expressed sequence tag approach was used to identify cDNAs for enzymes involved in the biosynthesis of $20{:}1\Delta^5$). By random sequencing of a library prepared from developing *Limnanthes douglasii* seeds, a class of cDNAs was identified that encode a homolog of acyl-coenzyme A (CoA) desaturases found in animals, fungi, and cyanobacteria. Expression of a cDNA for the *L. douglasii* acyl-CoA desaturase homolog in somatic soybean (*Glycine max*) embryos behind a strong seed-specific promoter resulted in the accumulation of Δ^5-hexadecenoic acid to amounts of 2% to 3% (w/w) of the total fatty acids of single embryos. Δ^5-Octadecenoic acid and $20{:}1\Delta^5$ also composed <1% (w/w) each of the total fatty acids of these embryos. In addition, cDNAs were identified from the *L. douglasii* expressed sequence tags that encode a homolog of fatty acid elongase 1 (FAE1), a β-ketoacyl-CoA synthase that catalyzes the initial step of very long-chain fatty acid synthesis. Expression of the *L. douglassi* FAE1 homolog in somatic soybean embryos was accompanied by the accumulation of C_{20} and C_{22} fatty acids, principally as eicosanoic acid, to amounts of 18% (w/w) of the total fatty acids of single embryos. To partially reconstruct the biosynthetic pathway of $20{:}1\Delta^5$ in transgenic plant tissues, cDNAs for the *L. douglasii* acyl-CoA desaturase and FAE1 were co-expressed in somatic soybean embryos. In the resulting transgenic embryos, $20{:}1\Delta^5$ and Δ^5-docosenoic acid composed up to 12% of the total fatty acids.

The seed oil of *Limnanthes* spp. is distinct from that of other plants because of its high content of C_{20} and C_{22} fatty acids with Δ^5 unsaturation (Miller et al., 1964; Phillips et al., 1971). The most abundant component of the seed oil of these plants is Δ^5-eicosenoic acid[2] ($20{:}1\Delta^5$), which accounts for 60% of the total fatty acids (Miller et al., 1964). The close position of the double bond of this fatty acid to the carboxy terminus results in chemical and physical properties that are not found in oleic acid ($18{:}1\Delta^9$), the primary monounsaturated fatty acid of the seed oil of most plant species. For example, $20{:}1\Delta^5$ is more oxidatively stable than $18{:}1\Delta^9$ (Isbell et al., 1999) and can be used as a precursor for the synthesis of industrial compounds such as δ-lactones (Erhan et al., 1993). The novel properties associated with $20{:}1\Delta^5$ make the seed oil of *Limnanthes* sp. desirable for use in cosmetics, surfactants, and lubricants (Hirsinger, 1989; Burg and Kleiman, 1991). Because its seed oil has these unique properties, meadowfoam (*Limnanthes alba*) is grown as an oilseed crop on limited acreage in the Pacific Northwest of the United States (Hirsinger, 1989).

The biosynthesis of $20{:}1\Delta^5$ has been studied previously by radiolabeling of developing meadowfoam seeds as well as by assay of cell-free homogenates of these seeds (Pollard and Stumpf, 1980; Moreau et al., 1981). From these studies, Pollard and Stumpf (1980) proposed a biosynthetic pathway for $20{:}1\Delta^5$ that consists of three metabolic steps: (a) a large flux of palmitic acid (16:0) from the plastid to the endoplasmic reticulum; (b) microsomal elongation of 16:0, presumably as a coenzyme A (CoA) ester, to eicosanoic acid (20:0); and (c) Δ^5 desaturation of 20:0 to form $20{:}1\Delta^5$. The latter two steps of this pathway are distinct from fatty acid elongation and desaturation reactions described in other species. For example, the elongation of 16:0 to a C_{20} fatty acid contrasts with the synthesis of C_{20} and C_{22} fatty acids commonly found in seeds of the Brassicaceae family, including Arabidopsis and oilseed rape (*Brassica napus*) (Kunst et al., 1992; Taylor et al., 1992). In these seeds, $18{:}1\Delta^9$ is used instead as the primary fatty acid substrate for the synthesis of very long-chain fatty acids (Kunst et al., 1992). This difference likely reflects the substrate specificity of fatty acid elongase 1 (FAE1), a β-ketoacyl-CoA synthase that catalyzes the initial condensation reaction in the synthesis of very long-chain fatty acids (Millar and Kunst, 1997). Therefore, the pathway proposed for $20{:}1\Delta^5$ formation in *Lim-*

[1] The Plant Biotechnology Institute portion of this research is partially supported by the Agri-Food Innovation Fund (project no. 96000414).

[2] Δ^z, Double bond is positioned at the zth carbon atom relative to the carboxyl end of the fatty acid.

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nanthes sp. seeds is most consistent with the presence of an FAE1 polypeptide that has greater specificity for CoA esters of 16:0 than for 18:1Δ^9.

In addition, based on in vitro assays of *Limnanthes* sp. seed extracts, Moreau et al. (1981) suggested that 20:0-CoA is the substrate for the Δ^5-desaturase. Although acyl-CoA desaturation is the major route of monounsaturated fatty acid synthesis in animals and fungi (Bloomfield and Bloch, 1960; Strittmatter et al., 1974), the use of acyl-CoAs as substrates for fatty acid desaturases has yet to be demonstrated in plants. In this regard, cDNAs for acyl-CoA desaturase-related polypeptides have been identified in several plant species; however, their functions have not been established (Fukuchi-Mizutani et al., 1995, 1998). Instead, plant desaturases have only been shown to date to use fatty acids bound to glycerolipids or acyl carrier protein as substrates (Shanklin and Cahoon, 1998). Therefore, the involvement of an acyl-CoA desaturase in the synthesis of 20:1Δ^5 would represent a novel pathway for unsaturated fatty acid formation in plants.

To further characterize the biosynthetic pathway of 20:1Δ^5 and to explore the possibility of producing 20:1Δ^5-containing oil in a domestic oilseed crop, an expressed sequence tag (EST) approach was undertaken. As described here, random sequencing of a cDNA library prepared from *Limnanthes douglasii* seeds resulted in the identification of cDNAs for a saturated fatty acid-specific FAE1 homolog and a Δ^5-desaturase that is most closely related to known acyl-CoA desaturases. Consistent with the predictions of Pollard and Stumpf (1980), we further demonstrate that the pathway for 20:1Δ^5 synthesis can be transferred to somatic soybean (*Glycine max*) embryos by co-expression of cDNAs for the *L. douglasii* Δ^5-desaturase and FAE1 homolog.

RESULTS

EST Analysis of Developing *L. douglasii* Seeds

An EST approach was used to identify cDNAs for enzymes involved in the biosynthesis of 20:1Δ^5. As part of this effort, nucleotide sequence was obtained from 400 to 500 bp of 1,145 random cDNAs in a library prepared from developing *L. douglasii* seeds. Given the pathway for 20:1Δ^5 synthesis proposed by Pollard and Stumpf (1980), homology searches of sequences from the *L. douglasii* cDNA library focused on the identification of ESTs for fatty acid desaturases and FAE1-related enzymes. In this regard, a class of cDNAs was identified that encodes portions of a polypeptide that is most related to acyl-CoA desaturases from animal, fungal, and cyanobacterial sources. This class was represented by five cDNAs of varying lengths. The partial 5′ sequences of these cDNAs shared 98% identity in regions of at least 100 bp of overlap. The longest cDNA of this class encoded a polypeptide of 356 amino acids, but contained no in-frame stop codon in its 5′ terminus. This polypeptide was found to share 20% to 25% amino acid sequence identity with Δ^9-acyl-CoA desaturases from rat (Thiede et al., 1986), human (Zhang et al., 1999), and *Saccharomyces cerevisiae* (Stukey et al., 1990) and 43% identity with the Δ^9-desaturase from the cyanobacteria *Anabaena variabilis* (Sakamoto et al., 1994) (Fig. 1). The *L. douglassi* polypeptide, however, was most related (45%–50% identity) to acyl-CoA desaturase-like polypeptides of unknown function from rose (Fukuchi-Mizutani et al., 1995) and Arabidopsis (Fukuchi-Mizutani et al., 1998). No other class of fatty acid desaturase ESTs was detected among the random sequences generated from the *L. douglasii* cDNA library.

In addition, three cDNAs encoding an FAE1-related polypeptide were detected among the random *L. douglasii* sequences. The 5′-terminal portions of these cDNAs from the raw EST data shared ≥97%

```
 LimDes ------------LRLSLYFPISISLSLSLEAMASFIATTTPAMPAFASVLDPKIPTKPEP  48
AraDes1 ------------------------------------------------------MS   2
AraDes2 ------------------------------------------------------MS   2
 AnaDes ------------------------------------------------------     0
HomoDes ----------------------MPAHL:QDDISS:YTT:::ITA:PPGVLQNGGDKLETM:  39
 SacDes MPTSGTTIELIDDQFPKDDSA:SGIVDEVDLTEAN:LA:GLNKK:PRI:NGFGSLMGSKE  60

 LimDes KTETPKPKDDLERFRTSEVVLERKAKG------------FWRRK-------WNPRDIQN  88
AraDes1 LSASE:-EENNKKMAADKAEMG::KR-------------AM:E::-------:KRL::VK  41
AraDes2 V:S:VE-ENHQKNPS:PAA:E:K:KRRW-----------V::D:R-------:RRL:YVK  43
 AnaDes ------------------MTIATST:P-------------QIN---------:VN---TL  17
HomoDes LYLEDDIRP:IKDDIYDPTYKDKEGPS------------PKVEYV-------:RN-I:LM  79
 SacDes MVSVEFD:KGN:KKSNLDRL::KDNQEKEEAKTKIHISEQP:TLNNWHQHLN:LN-MVLV 119

 LimDes AVTLLVLHALAAMAPFYFSWDAFWISFILLGFASGVLGITLCFHRCLTHGGFKLPKLVEY 148
AraDes1 :FAS:FV:F:CLL:::N:T:P:LRVAL:VY--TV:G::::VSY::N:A:RS::V::WL::  99
AraDes2 FSASFTV:S::LL::::T:S:L:VT:LFY--TI:G::::VSY::N:A:RS::V::WL::  101
 AnaDes FFLG:HIG::F:FI:SN:::A:VGVALL:Y-WIT:G::::G:::LV::RS:QT::WL::  76
HomoDes SLLH:GALYGITLI:TCKEYTWL:GV:YYF---VSA::::AGA::LWS:RSY:ARLPLRL 136
 SacDes CGMPMIGWYF:LSGKVPLHLNV:LF:VFYY--:V:GVS::AGY::LWS:RSYSAHWPLRL 177

 LimDes FFAYCGSLALQGDPMEWVSNHRYHHQFVDTERDVHSPTQGFWFCHIGWVLDKDLFVEKRG 208
AraDes1 ::::::L::I::::ID:::T:::::::T:SD::P:::NE::::S:LL:LF:TGYL:::C: 159
AraDes2 LL:::AL::I::::ID:::T:::::::T:S:::P:::KE::::S:LL:IY:SAYL:S:C: 161
 AnaDes :LVL::T::C::G:I:::GT::I::LHS::DP:P:DSNK:::WS::::LIYHSPSHADVP 136
HomoDes :LIIANTM:F:N:VY::ARD::A::K:SE:HA:P:NSRR::F:S:V::L:VRKHPAV:EK 196
 SacDes :Y:IF:CASVE:SAKW:GHS::I::RYT::L::PYDARR:L:YS:M::M:L:PNPKY:AR 237

 LimDes GRRNNVNDLKKQAFYRFLQKTYMYHQLALIALL-----YYVGGFPY---IVWGMGFRLVF 260
AraDes1 -::T::E:::R:WY:K:::R:VL::I:TFGF::-----::F::LSF---LT::::IGVAM 210
AraDes2 -::A::E:::R:W:::::::VLF:I:G:GFF:-----F:L::MSF---VT::::VGAAL 212
 AnaDes ---RFTK:IAEDPV:Q::::YFIFI:I::GL::-----L:L::WSF---V::::VF::I:W 185
HomoDes :STLDLS::EAEKLVM:QRRY:KPGL:MMCFI:PTLVPW:FW:ETFQNSVFVATFL:YAV 256
 SacDes ---ADIT:MTDDWTI::QHRH:ILLM:LTAFVIPTLICG:FFNDYMGG-LIYAGFI:VFV 293

 LimDes MFHSTFAINSVCHKWGGRPWNTGDLSTNNMFVALCAFGEGWHNNHHAFEQSARHGLEWWE 320
AraDes1 EH:V:CL:::L::V::S:T:K:N:T:R:VWWLSVFS:::S:::::::::S:::Q:::::Q 270
AraDes2 EV:V:CL:::L::I::T:T:K:N:T:R:VWWLSVFS:::S:::::::::S:::Q:::::Q 272
 AnaDes VY:C:WLV::AT::F:Y:TYDA::R:::CWW::VLV:::::::::::::QY:::::::::: 245
HomoDes VLNA:WLV::AA:LF:Y::YDKNISPRE:IL:S:G:V:::F::Y::S:PYDYSASEYR:H 316
 SacDes IQQA::C:::MA:YI:TQ:FDDRRTPRD:WIT:IVT::::Y::F::E:PTDY:NAIK:YQ 353

 LimDes IDVTWYVIRTLQAIGLATNVKLPTEAQKQKLKAKSA-------                  356
AraDes1 ::IS::IV:F:EI:::::D::::S:S:RRRMAMVR--------                  305
AraDes2 ::IS::IV:FFEI:::::D::V:::::RRRMAIVR--------                  307
 AnaDes V:L::MTVQL::IL:::::::::ADKK:----------------                  272
HomoDes :NFNTFF:DWMA:L::TYDR:KVSK:AILARIKRTGDGNYKSG                  359
 SacDes Y:P:KVI:YLTSLV:::YDL:KFSQNAIEEALIQQEQKKIN--                  394
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Figure 1. Comparison of the amino acid sequences of the *L. douglasii* acyl-CoA desaturase homolog (LimDes; accession no. AF247133) with those of related polypeptides from plant, mammalian, cyanobacterial, and fungal sources. The alignment contains the sequences of Arabidopsis (AraDes1 and AraDes2) desaturase homologs as well as the sequences of Δ^9-acyl-CoA desaturases from human (HomoDes), *S. cerevisiae* (SacDes), and *A. variabilis* (AnaDes). Colons indicate residues that are identical to those in the LimDes sequence and alignment gaps are indicated by dashes. The GenBank accession numbers for the sequences shown are AF247133 (LimDes), D88536 (AraDes1), D88537 (AraDes2), D14581 (AnaDes), AF097514 (HomoDes), and J05676 (SacDes). (The C-terminal cytochrome b_5 domain is not included in the *S. cerevisiae* sequence.)

identity over more than 200 bp of overlapping sequence. The longest of these cDNAs encoded a polypeptide with 506 amino acids that was most related to an FAE1 from seeds of jojoba (*Simondsia chinensis*) (66% amino acid sequence identity) (Lassner et al., 1996) (Fig. 2). The *L. douglasii* polypeptide also shared 50% identity with FAE1 from Arabidopsis (James et al., 1995) and oilseed rape (Clemens and Kunst, 1997) seeds and approximately 55% identity with the Arabidopsis KCS1 (Todd et al., 1999) and CUT1 (Millar et al., 1999) polypeptides. The latter enzymes are β-keotacyl-CoA synthases that are involved in the synthesis of very long-chain fatty acids for leaf cuticular wax (Millar et al., 1999; Todd et al., 1999).

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LimFAE MSETKPEKPLI--------ATVKNTLPDLKLSINLKHVKLGYHY---LITHGMYLCLPPLAL  51
BrasFAE :T-----------------------------:::---:::L:::---V::NLFN:::FF::TA  27
AraFAE :T-----------------------------:V:---:::L:R:---VL:NFFN:::F::TA  27
AraCUT1 :PQAP---------------------N:EFSS:VK::Y:::::Q:---:VN:FLSFL:I:IMA  39
SimFAE :KAKTITN:E:QVSTTMTTT:TTA:::NF:S:::H:::::::---:::SNALF:VFI::LG  59
AraKCS :DRERLTAEMAFRDSSSAVIRIRRR:::LT:VK::Y::::L:NSCNVT:ILFF:IIL::TG  62

LimFAE VLFAQISTLSLKDFNDIW--EQLQFNLISVVVSSTLLVSLLILYFMTRPRPVYLMDFACYKP 111
BrasFAE IVAGKAY-:TID:LHHLYYS-Y::H:::TIAPLLAFT:FGSV::IA:::K:::VEYS::L:  87
AraFAE F:AGKA:R:TIN:LHN-FLS-Y::H:::T:TLLFAFT:FG:V::IV:::N:::V:YS::L:  87
AraCUT1 IVAVELLRMGPEEILNV:NS--:::D:VQ:LC::FFVIFISTV::SK::TI::V:YS:::  99
SimFAE LAS:HL:SF:AH:LSLLF--DL:RR::LP:::C:F:F:L:AT:H:L:::N:::V:::::::  119
AraKCS TVLV:LTG:TFDT:SEL:SNQAV:LDTATRLTCLVF:SFV:T::VAN:SK:::V::S::::  124

LimFAE DETRKSTREHFMK----------CGESLGSFTEDNIDFQRKLVARSGLGDATYLPEAIGTIP 163
BrasFAE PTHCR:SISKV:DIFFQVRKAD-PSRNGTCDDSSWL::L::IQE:::::E:HG::GLLQV:  148
AraFAE PPHL:VSVSKV:DIFYQIRKADTSSRNVACDDPSSL::L::IQE:::::E::S::GLIHV:  149
AraCUT1 PV:CRVPFAT::E----------HSRLILKDKPKSVE::MRILE:::::EE:C::P::HY::  151
SimFAE HPNLITSH:M::D----------RTSRA:::SKE::E::::ILE:A:M:RE::V:KSVTKV:  171
AraKCS EDE::ISVDS:LT----------MT:EN:::D:TVQ::QRISN:A::::E:::RG:TST:  176

LimFAE AHPSMKAARREAELVMFGAIDQLLEKTKVNPKDIGILVVNCSLFSPTPSLSSMIVNHYKLRG 225
BrasFAE PRKTFAR::E:T:Q:II::LEN:FKN:N:::::::::::S:M:N:::::A:V::TF:::S  210
AraFAE PRKTFA:S:E:T:K:II::LEN:F:N:::::RE:::::::S:M:N:::::A:V::TF:::S  211
AraCUT1 PT:T:D:::S::QN:I:E:M:D:FK::GLK:::VD::I:::::::::::::A:VI:K:::S  213
SimFAE PE::IA:::A:::E::Y::::EV:::G:K::Q:::::::::::N:::::::::::::::::  233
AraKCS PKLN:SE::A:::A:::::L:S:P:::GIK:AEV:::I:::::N:::::A:::::::M:E  238

LimFAE NIISYNLGGHGCSAGLISVDLAKRLLETNPNTYALVMSTENITLNWYMGNDRSKLVSNCLFR 287
BrasFAE :VR:F:::::::::V:AI::::D::HVHK:::::V:::::Y:I:A:DN::MM:::::::  272
AraFAE ::K:F:::::::::V:AI::::D::HVHK:::::V:::::QGI:A:EN::MM:::::::  273
AraCUT1 ::K:F::S:::::::::::::RD::QVH::SN:IIV:::I::P:Y:Q::E:AM:LP:::::  275
SimFAE ::L:::::::::::::::I:::D::QVYR:::V::V:::M:::::N:::::M:IT:::::  295
AraKCS D:K:::::::::::::::I:::NN::KA:::S::V:V:::::::::F:::::M:LC::I::  300

LimFAE MGGAAVLLSNKTSDKKRSKYQLVTTVRSHKGADDNCYGCIFQEEDSNGKIGVSLSKNLMAVA 349
BrasFAE V::::I:::::PR:RR:::E::H:::T:T:::KSFR:VQ:GD:E::QT:::::DITD::  334
AraFAE V::::I:::::SG:RR:::K::H:::T:T:::KSFR:VQ::D:ES:::::C::DITN::  335
AraCUT1 ::A:::IHM:::RR::RW:A::K:SHL::T:R:::KSFY:VYEQ::KE:HV:IN:::D:::I:  337
SimFAE :::::II:::RWR:RR:::::LH:::T:::::KS:R:VL:Q::E:N:V::A:::D:::::  357
AraKCS :::::I::::RRQ:R:K::::S::NV:::T:::S::KN:N:VY:K::ER:T:::::ARE::S::  362

LimFAE GDALKTNITTLGPLVLPMSEQLLFFATLVARKVFKK-KIKPYIPDFKLAFDHFCIHAGGRAV 410
BrasFAE :RTV:K::A:::::I::L::K:::::V:FMGK:L::DEIKHY:V:::::::I:::::::::K::  396
AraFAE :TT:TK::A:::::I::L::KF:::::F::K:LL:DKIKHY:V:::::V:::::::::::  397
AraCUT1 :E:::A:::I:::::A:::::LTS:IG:::I:N-P:N:::::::::::E:::::::::  398
SimFAE :E:::A:::::::::::::::::::::::::::::MTNV:::::::::AK:::::::K::  419
AraKCS ::::::::::::::M:::L::::M:LIS::K:M::L-V:::::::::::E:::::::::  423

LimFAE LDELEKNLQLSSWHLEPSRMTLYRFGNTSSSSLWYELAYSEAKGRIRKGERVWQIGFGSGFK 472
BrasFAE I:V::::G:APIDV:A::S::H:::::::::I::::::I:P:::MK::NK:::AL:::::  458
AraFAE I:::::::G::PIDV:A::S::H:::::::::I::::::I:::::MK::NKA:::AL:::::  459
AraCUT1 I:::Q::::::GE:V:A::::::H:::::::::::::::S:I:S:::M:R:D::::::A::::::  460
SimFAE :::::T::E:TP:::::::::::::::::::::::::::::::A:::::::::D:T:M:::::::  481
AraKCS :::VQ:::D:KD::M:::::::H:::::::::::::::M:::T::::VKA:D:L:::A::::::  485

LimFAE CNSAVKKALKSVDPKKEK-NPWMDEIHQPPV---------------AVV-                505
BrasFAE ::::::V::NN:KAS--TNS::EHC:DRY::KIDSDSGKSETRVPNGRS                505
AraFAE ::::::V::RN:KAS--ANS::QHC:DRY::KIDSDLSKSKTHVQNGRS                506
AraCUT1 :::::::CNRTIKTP--:DG::S:C:DRY::FI--------PEV--:KL               497
SimFAE :::V::R::R::N:AR::-:::::::EN:::HV--------PKIAPIAS                521
AraKCS :::::::::RP:STEEMTG:A:AGS:D:Y::KV--------------:Q                 520
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Figure 2. Comparison of the amino acid sequences of the *L. douglasii* FAE1 homolog (LimFAE; accession no. AF247134) with those of FAE1 polypeptides and related β-ketoacyl-CoA synthases from other plant species. The alignment contains the sequences of the Arabidopsis (AraFAE), oilseed rape (BrasFAE), and jojoba (SimFAE) FAE1 polypeptides. Also shown are two Arabidopsis β-ketoacyl-CoA synthases whose activities are associated with epicuticular wax synthesis (AraKCS and AraCUT1). Colons indicate residues that are identical to those in the LimFAE sequence, and sequence alignment gaps are maintained with dashes. The GenBank accession numbers for the sequences shown are AF247134 (LimFAE), U29142 (AraFAE), AF009563 (BrasFAE), U37088 (SimFAE), AF053345 (AraKCS), and AF129511 (AraCUT1).

Expression of *L. douglasii* Acyl-CoA Desaturase and FAE1 Homologs in Somatic Soybean Embryos

To establish their functional identity, cDNAs for the acyl-CoA desaturase- and FAE1-related polypeptides obtained from the EST screen were expressed in somatic soybean embryos. Like seeds, somatic soybean embryos accumulate triacylglycerols, and the fatty acid composition of transgenic embryos has been shown to be completely predictive of the fatty acid composition of seeds from plants regenerated from embryos (Kinney, 1996). For these experiments, expression of cDNAs was placed under the control of the strong seed-specific promoter of the α′-subunit of β-conglycinin (Doyle et al., 1986).

In the case of the *L. douglasii* acyl-CoA desaturase homolog, the coding sequence for amino acids 31 through 356 (as shown in Fig. 1) was expressed in somatic soybean embryos. This expression resulted in the accumulation of several monounsaturated fatty acids that were not detected in untransformed embryos (Fig. 3). These fatty acids were identified by GC-MS analysis of dimethyl disulfide derivatives of their methyl esters as the Δ^5 isomers of hexadecenoic (16:1), octadecenoic (18:1), and eicosenoic (20:1) acids (results not shown). The most abundant of these fatty acids was 16:1Δ^5, which accounted for 2% to 3% (w/w) of the total fatty acids of single embryo samples (Table I). The Δ^5 isomers of 18:1 and 20:1 each composed <1% of the total fatty acids of the transgenic soybean embryos. Trace amounts of Δ^5-docosenoic acid (22:1Δ^5) were also detected (as confirmed by GC-MS) in some of the transgenic embryos. However, no Δ^5-polyunsaturated fatty acids were found in extracts of transgenic embryos. Overall, the identification of a series of Δ^5-monounsaturated fatty acids in transgenic somatic soybean embryos provided conclusive evidence that the *L. douglasii* acyl-CoA desaturase homolog functions as a Δ^5-desaturase.

Expression of a full-length cDNA for the *L. douglasii* FAE1 homolog in somatic soybean embryos resulted in the accumulation of C_{20} and C_{22} fatty acids (Fig. 3C). These fatty acids were found to collectively account for 18% (w/w) of the total fatty acids of single transgenic embryos (Table I). In contrast, C_{20} and C_{22} fatty acids typically compose <1% of the fatty acids of untransformed somatic soybean embryos. The major component of the mixture of very long-chain fatty acids in transgenic embryos was 20:0, which composed nearly 13% (w/w) of the fatty acids of single embryos. In addition, lesser amounts of 20:1 (Δ^{11}- and Δ^{13}-isomers), eicosadienoic acid (20:2), and docosanoic acid (22:0) were detected in embryos transformed with the *L. douglasii* FAE1

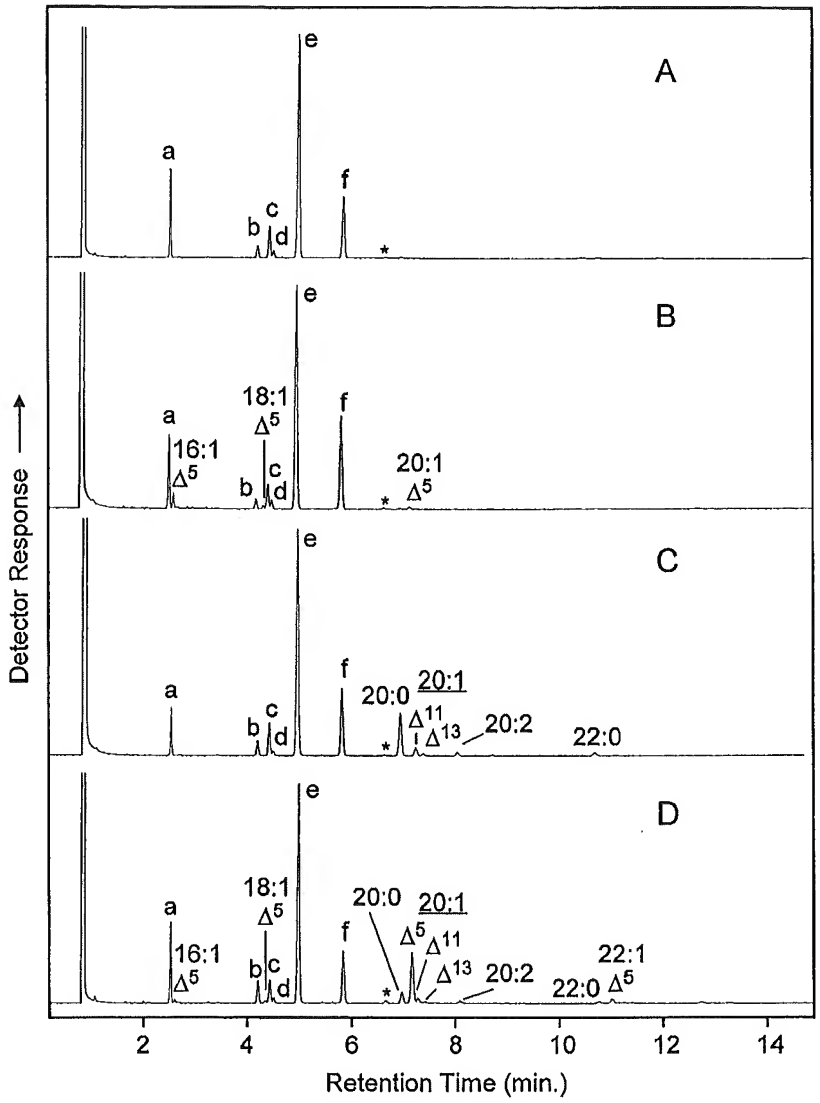

Figure 3. Gas chromatographic analyses of fatty acid methyl esters prepared from an untransformed somatic soybean embryo (A) and transgenic embryos expressing the *L. douglasii* acyl-CoA desaturase homolog (B) and the *L. douglasii* FAE1 homolog (C). D contains a gas chromatogram of fatty acid methyl esters prepared from a transgenic somatic soybean embryo cotransformed with cDNAs for the *L. douglasii* acyl-CoA and FAE1 homologs. Peaks labeled a through f correspond to fatty acids found in all samples. The identities of these fatty acids are: a, 16:0; b, stearic acid (18:0); c, 18:1Δ^9; d, cis-vaccenic acid (18:1Δ^{11}); e, linoleic acid (18:2$\Delta^{9,12}$); and f, α-linolenic acid (18:3$\Delta^{9,12,15}$). The peak labeled with an asterisk corresponds to phytol, as determined by gas chromatography-mass spectrometry (GC-MS) analysis. Amounts of this compound detected in extracts of somatic soybean embryos correlate with their chlorophyll content.

homolog. It is interesting that the accumulation of C_{20} and C_{22} fatty acids appeared to occur at the expense of 16:0 in transgenic embryos. In this regard, amounts of 16:0 declined from approximately 15% (w/w) in untransformed embryos to as little as 6% to 7% (w/w) in transgenic embryos with the highest content of C_{20} and C_{22} fatty acids (Fig. 4).

Co-Expression of *L. douglasii* Acyl-CoA Desaturase and FAE1 Homologs in Somatic Soybean Embryos

The alterations in fatty acid composition resulting from the expression of the *L. douglasii* acyl-CoA desaturase and FAE1 strongly suggested that these enzymes are components of the 20:1Δ^5 biosynthetic pathway. To further examine the involvement of these enzymes in 20:1Δ^5 biosynthesis, cDNAs encoding the acyl-CoA desaturase and FAE1 homologs were co-expressed in somatic soybean embryos. In this experiment, the coding sequences for the two polypeptides were placed behind the promoter of the gene for the α′-subunit of β-conglycinin on separate plasmids. The plasmid carrying the FAE1 cDNA contained a hygromycin resistance gene for selection of transgenic plant material, while the plasmid containing the acyl-CoA desaturase cDNA lacked a plant selection marker. The two expression plasmids were then cobombarded into somatic soybean embryos, using a 10:1 molar ratio of plasmid carrying the acyl-CoA desaturase cDNA:plasmid carrying the FAE1 cDNA. One of the resulting transgenic events (MS251-2-11) displayed a phenotype consistent with the activities of both enzymes (Fig. 3D). In addition, expression of both cDNAs in this event was confirmed by PCR amplification using sequence-specific primers and first-strand cDNA prepared from total RNA isolated from transgenic embryos. In single embryos from event MS251-2-11, Δ^5-monounsaturated

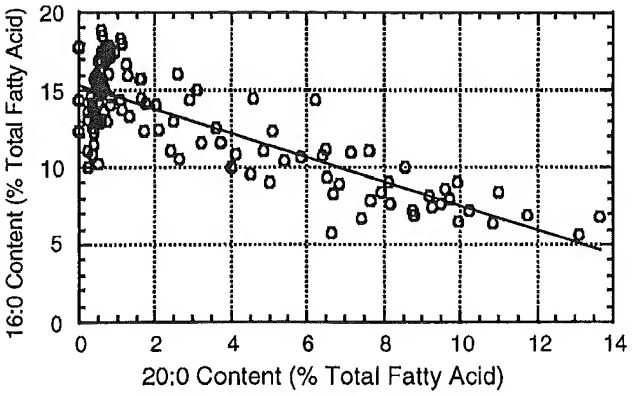

Figure 4. Comparison of the content of 20:0 and 16:0 acids in transgenic somatic soybean embryos expressing the *L. douglasii* FAE1 homolog. Amounts of 16:0 and 20:0 are expressed as their weight % of the total fatty acids of single embryo samples. The plotted values are derived from fatty acid compositional analyses of 122 single embryos from 30 transformation events ($R = 0.82$).

fatty acids were found to accumulate to nearly 13% of the total fatty acids. In addition, C_{20} and C_{22} fatty acids accounted for approximately 19% of the total fatty acids of these embryos. Nearly all of the Δ^5-fatty acids were detected in the form of 20:1Δ^5 (10.8% of the total fatty acids) and 22:1Δ^5 (1.3% of the total fatty acids) (Table I). The double-bond position of these fatty acids was confirmed by GC-MS analysis as shown in Figure 5. No Δ^5 polyunsaturated fatty acids were detected in extracts from the transgenic embryos. Similar to what was observed with the expression of the FAE1 homolog alone, the 16:0 content decreased from approximately 15% in untransformed embryos to 9% in soybean embryos co-expressing the acyl-CoA desaturase and FAE1 cDNAs.

DISCUSSION

The pathway for 20:1Δ^5 synthesis in *Limnanthes* sp. seeds was previously proposed to contain a fatty acid elongation system that converts 16:0, presumably as a CoA ester, to 20:0 and a Δ^5-acyl-CoA desaturase that converts 20:0-CoA to 20:1Δ^5-CoA (Pollard and Stumpf, 1980; Moreau et al., 1981). Using an EST strategy, we have identified cDNAs from *L. douglasii* that when expressed in somatic soybean embryos yield alterations in fatty acid composition consistent with this pathway. In this regard, a class of cDNAs was identified among the *L. douglasii* ESTs for a β-ketoacyl-CoA synthase with close relation to FAE1 from seeds of the Brassicaceae family (James et al., 1995; Clemens and Kunst, 1997) and jojoba (Lassner et al., 1996). The in vivo activity of the *L. douglasii* enzyme, however, differed from that previously described for FAE1 polypeptides from Brassicaceae seeds, which are associated with the preferential elongation of monounsaturated fatty acids (Kunst et al., 1992; Taylor et al., 1992). In contrast, expression of the *L. douglasii* FAE1 homolog resulted primarily in the accumulation of saturated very long-chain fatty acids, principally in the form of 20:0. In addition, the relative content of 16:0 in transgenic embryos accumulating the greatest amounts of 20:0 was more than 2-fold lower than that detected in untransformed embryos. These findings are thus consistent with 16:0 serving as the initial substrate for 20:1Δ^5 synthesis in *L. douglasii* seeds via an elongation pathway that contains a saturated fatty acid-specific FAE1, as previously proposed (Pollard and Stumpf, 1980).

In addition, we have identified cDNAs among the pool of ESTs from developing *L. douglasii* seeds for a polypeptide that is structurally related to acyl-CoA desaturases from animals, yeast, and cyanobacteria. Expression of this polypeptide in somatic soybean embryos was found to result in the accumulation of Δ^5-monounsaturated fatty acids. Therefore, this result agrees with the suggestion of Moreau et al. (1981) that the Δ^5-desaturase in *Limnanthes* sp. seeds is an acyl-CoA-type fatty acid desaturase. Our finding that the acyl-CoA-like desaturase of *L. douglasii* is a functional Δ^5-desaturase is the first demonstration of the activity of an acyl-CoA-related desaturase in plants. In this regard, the occurrence of cDNAs for acyl-CoA desaturase-like polypeptides has been reported in several plant species, including Arabidopsis and rose, but functions have not yet been demonstrated for these enzymes (Fukuchi-Mizutani et al., 1995; Fukuchi-Mizutani et al., 1998). It remains to be confirmed experimentally that the actual substrate of the *L. douglasii* acyl-CoA desaturase-related enzyme is indeed acyl-CoA and not, for example, a polar lipid. However, in terms of the acyl group itself, our results from transgenic soybean embryos do confirm that the *L. douglasii* Δ^5-desaturase has a marked sub-

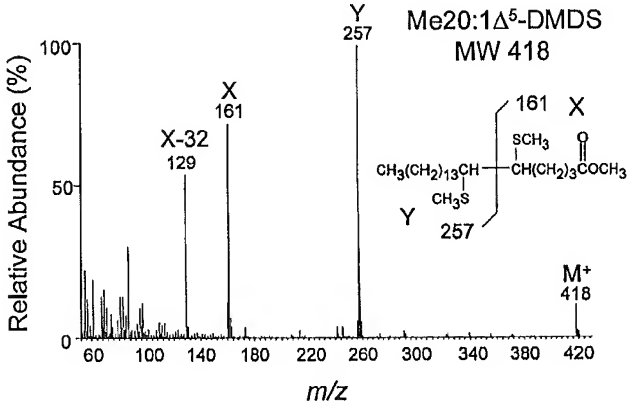

Figure 5. Mass spectral identification of 20:1Δ^5 from somatic soybean embryos co-expressing the *L. douglasii* acyl-CoA desaturase and FAE1 homologs. The mass spectrum shown was obtained by GC-MS analysis of the dimethyl disulfide derivatives of unsaturated fatty acid methyl esters prepared from transgenic soybean embryos. The mass spectrum of the dimethyl disulfide derivative of methyl 22:1Δ^5 from extracts of these embryos contained a molecular ion (M+) of 446 *m/z* as well as X, X-32, and Y fragments of 161, 129, and 285 *m/z*, respectively (data not shown).

strate specificity for 20:0. This specificity is evidenced by the higher amounts of Δ^5-fatty acids, principally in the form of 20:1Δ^5, obtained by co-expression of the Δ^5-desaturase and FAE1. Results obtained from the expression of the *L. douglasii* Δ^5-desaturase alone indicate that this enzyme is also capable of functioning on other saturated fatty acids, including 16:0 and 18:0, in the absence of significant substrate pools of 20:0. Overall, the in vivo properties of the *L. douglasii* Δ^5-desaturase are in general agreement with the in vitro substrate specificity profile previously reported for this enzyme in *L. alba* seed extracts (Moreau et al., 1981).

In spite of our demonstration of cDNAs for two enzymatic components of the 20:1Δ^5 biosynthetic pathway, it is likely that other metabolic factors are required for high levels of synthesis and accumulation of this fatty acid. Foremost among these factors is likely to be an enzyme(s) that generates a large microsomal pool of 16:0 to drive flux into the 20:1Δ^5 biosynthetic pathway. A candidate for such an enzyme is an acyl-ACP thioesterase such as FatB that releases 16:0 from de novo fatty acid synthesis in the plastid for export to the cytosol (Dörmann et al., 1995). It would be predicted that the overexpression of a FatB-type enzyme would result in an increased flux of 16:0 into the synthesis of 20:1Δ^5. The combined effect of the over-expression of FatB, together with the *L. douglassi* Δ^5-desaturase and FAE1, would thus likely yield amounts of 20:1Δ^5 in excess of the amount reported here. It is also conceivable that to achieve the highest amounts of 20:1Δ^5 in soybean, additional *L. douglasii* enzymes, such as acyltransferases, might be necessary. Finally, it should also be noted that the Δ^5-desaturase cDNA expressed in transgenic soybean embryos in this study is probably not full-length. We subsequently cloned a longer cDNA which encoded a Met-20 upstream of Met-31 in the truncated clone. It is likely that Met-20 is the actual start Met of this gene. Although the truncated Δ^5-desaturase was clearly active in transgenic soybean embryos, it is possible that the absence of a complete polypeptide might result in some reduction in the in vivo specific activity of this enzyme.

Limnanthes sp. seed oil also contains significant proportions of erucic acid (22:1Δ^{13}) (15%–20%) and an unusual diene 22:2 $\Delta^{5,13}$ (10%–20%) (Miller et al., 1964; Phillips et al., 1971). Because of the large distance between its double bonds, 22:2$\Delta^{5,13}$ has potential industrial utility in the production of novel estolides and hydroxy fatty acids (Burg and Kleiman, 1991; Erhan et al., 1993). As proposed by Pollard and Stumpf (1980), the pathway of 22:2$\Delta^{5,13}$ synthesis appears to involve elongation of 18:1Δ^9-CoA to produce 20:1Δ^{11} and 22:1Δ^{13} in a manner similar to that found in Brassicaceae seeds (Kunst et al., 1992; Taylor et al., 1992). The $\Delta^{5,13}$ isomer of 22:2 was suggested to be formed by further desaturation of 22:1Δ^{13} at the Δ^5-position, presumably by the same acyl-CoA desaturase responsible for the synthesis of 20:1Δ^5 (Pollard and Stumpf, 1980). The lack of significant 22:1Δ^{13} accumulation upon expression of the *L. douglasii* FAE1 homolog described here suggests the likelihood of a second FAE1 in *L. douglasii* seeds that is more specific for the elongation of 18:1Δ^9. Based on this, we would predict that production of 22:1$\Delta^{5,13}$ in a transgenic plant would require the additional expression of a Brassicaceae-type FAE1 to generate sufficient substrate pools of 22:1Δ^{13} for the Δ^5-desaturase. In summary, the results described here show that the pathway for 20:1Δ^5 biosynthesis may be transferred to other species and demonstrate the possibility of producing a meadowfoam-type seed oil in transgenic crops.

MATERIALS AND METHODS

Construction of a cDNA Library from Developing Seeds of *Limnanthes douglasii*

Cotyledons dissected from developing seeds of *L. douglasii* were used for the construction of a cDNA library. For isolation of total RNA, 1.4 g of frozen *L. douglasii* cotyledons were ground to a fine powder and transferred to 12 mL of an extraction buffer containing 1 M Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 8.0), 1% (w/v) sodium dodecyl sulfate, 20 mM EDTA (pH 8.0), and 5% (v/v) β-mercaptoethanol and an equal volume of phenol:chloroform (1:1, v/v). Following centrifugation, the aqueous layer was re-extracted with phenol:chloroform (1:1, v/v) and subsequently extracted with chloroform:isoamyl alcohol (24:1, v/v). Lithium chloride was then added to the recovered aqueous layer to a final concentration of 2 M. Following precipitation on ice for 2 h, total RNA was collected by centrifugation and resuspended in water. The total RNA was reprecipitated with the addition of sodium acetate (pH 5.0) to a concentration of 300 mM and 2.5 volumes of ethanol. The resulting total RNA obtained by centrifugation was used for the isolation of poly(A^+)-enriched RNA using the PolyATract mRNA Isolation Kit (Promega, Madison, WI) according to the manufacturer's protocol.

First strand cDNA was prepared from *L. douglasii* sp. poly(A^+)-enriched RNA using avian myeloblastosis virus reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dT) primer that contained *Not*I recognition sequence at its 3′ terminus. Following synthesis of second strand cDNA with DNA polymerase I and blunting with T4 DNA polymerase, *Bst*XI/*Eco*RI adaptors (Invitrogen) were ligated onto the double-stranded cDNAs. The cDNAs were then selected by size on an agarose gel to remove cDNAs that were <500 bp. The size-selected cDNAs were then ligated bidirectionally into the *Bst*XI sites of the vector pcDNA2.1 (Invitrogen). The resulting cDNA library in plasmid form was maintained in the *Escherichia coli* strain TOP10F′ and stored as glycerol stocks at −80°C until used in expressed sequence tag (EST) analysis.

EST Analysis of cDNAs from Developing *L. douglasii* Seeds

Plasmids for EST analysis were prepared from randomly picked colonies from the *Limnanthes* sp. cDNA library in *E. coli* TOP10F′ cells using the R.E.A.L. Prep 96 System (Qiagen USA, Valencia, CA) according to the manufacturer's protocol. The sequencing methodology and public database sequence comparisons of the resulting ESTs were the same as described elsewhere (Cahoon et al., 1999), except that the T7 primer was used for sequencing of cDNAs.

Expression of *Limnanthes* sp. cDNAs in Somatic Soybean Embryos

A cDNA encoding amino acids 31 through 357 of the *L. douglasii* acyl-CoA desaturase homolog (see Fig. 1) was used for the preparation of plasmids for expression in somatic soybean embryos. The cDNA insert was initially cloned into the *Sma*I/*Xba*I sites of the vector pCST2 behind the promoter for the α′-subunit of β-conglycinin (Doyle et al., 1986). The resulting plasmid was designated pKS61. In addition to the promoter elements, the vector pCST2 contains a phaseolin termination sequence that flanks the 3′ end of cDNA inserts. A cassette from pKS61 containing the promoter fused with the *L. douglasii* cDNA and the flanking termination sequence was inserted as a *Hind*III fragment into the corresponding sites of pZBL100 to generate the plasmid pKS77. The vector pZBL100 contains a hygromycin B phosphotransferase gene behind the T7 RNA polymerase promoter for bacterial selection. This vector also contains a second hygromycin B phosphotransferase gene behind the cauliflower mosaic virus 35S promoter for selection of transgenic plant material.

For experiments involving the co-expression of cDNAs for the *L. douglasii* acyl-CoA desaturase and FAE1 homologs, the *Hind*III expression cassette from pKS61 was inserted into the corresponding sites of pKS17 to generate the plasmid pKS92. The vector pKS17 is essentially the same as pZBL100 except that it lacks the hygromycin resistance marker for transgenic plant selection.

A cDNA encoding a full-length *L. douglasii* FAE1 homolog from the EST analysis was cloned as a *Not*I fragment into the soybean expression vector pKS67 behind the promoter for the α′-subunit of β-conglycinin to generate the plasmid pLimFAE1. The vector pKS67, which has been described previously (Cahoon et al., 1999), contains hygromycin resistance markers for both bacterial and plant selection.

Somatic embryos of soybean (*Glycine max* cv Asgrow A2872) were transformed with expression constructs containing the cDNAs for the *L. douglasii* acyl-CoA desaturase and FAE1 homologs using particle bombardment as described previously (Finer and McMullen, 1991; Cahoon et al., 1999). Experiments involving the co-expression of cDNAs for *L. douglasii* acyl-CoA desaturase and FAE1 homologs were conducted by simultaneously bombarding somatic soybean embryos with plasmids pKS17 and pLimFAE1 at a molar ratio of 10:1. Transgenic embryos were selected and maintained as described (Finer and McMullen, 1991; Cahoon et al., 1999).

Expression of the *L. douglasii* acyl-CoA desaturase and FAE1 cDNAs in the reported transformation events was

Table I. *Fatty acid composition of somatic soybean embryos of untransformed lines and transgenic lines expressing cDNAs for the L. douglasii acyl-CoA desaturase (+Acyl-CoA desaturase), fatty acid elongase 1 (+FAE1), or co-expressing cDNAs for both the acyl-CoA desaturase and fatty acid elongase 1 (+Acyl-CoA Desaturase/+FAE1)*

Compositional data were obtained from three to five separate measurements (±SD) of single embryos from transformation events described in "Materials and Methods."

Fatty Acid	Untransformed (n = 3)	+Acyl-CoA Desaturase (n = 3)	+FAE1 (n = 5)	+Acyl-CoA Desaturase/ +FAE1 (n = 3)
		% total fatty acid (w/w)		
16:0	15.6 ± 1.4	11.9 ± 0.6	7.0 ± 1.2	9.2 ± 1.8
16:1Δ^5	ND[a]	2.4 ± 0.1	ND	0.3 ± 0.1
18:0	2.8 ± 0.3	2.1 ± 0.2	3.3 ± 0.4	3.7 ± 0.8
18:1Δ^5	ND	0.6 ± 0.1	ND	0.3 ± 0.1
18:1Δ^9/Δ^{11}[b]	8.2 ± 1.4	7.9 ± 0.7	7.3 ± 0.9	6.3 ± 0.8
18:2$\Delta^{9,12}$	51.4 ± 3.5	49.9 ± 0.6	48.4 ± 1.8	44.5 ± 1.1
18:3$\Delta^{9,12,15}$	20.1 ± 4.2	21.9 ± 0.6	15.7 ± 1.6	15.2 ± 3.5
20:0	0.5 ± 0.1	0.3 ± 0.1	12.5 ± 0.7	3.2 ± 0.8
20:1Δ^5	ND	0.7 ± 0.1	ND	10.8 ± 1.6
20:1Δ^{11}/Δ^{13}[c]	ND	ND	2.9 ± 0.3	2.0 ± 0.3
20:2$\Delta^{11,14}$	ND	ND	1.2 ± 0.2	1.1 ± 0.4
22:0	0.4 ± 0.1	0.3 ± 0.1	1.3 ± 0.2	0.7 ± 0.1
22:1Δ^5	ND	<0.2	ND	1.3 ± 0.1
Total Δ^5-fatty acids	ND	3.8	ND	12.7
Total ≥C_{20} fatty acids	0.9	1.4	17.9	19.1

[a] ND, Not detected. [b] Total amount of 18:1Δ^9 and 18:1Δ^{11}. [c] Total amount of 20:1Δ^{11} and 20:1Δ^{13}.

confirmed by PCR amplification using sequence specific primers and first-strand cDNA prepared from total RNA isolated from the transgenic somatic soybean embryos.

Fatty Acid Analysis of Transgenic Somatic Soybean Embryos

Fatty acid methyl esters were prepared from transgenic soybean embryos by homogenization of single embryos in 400 μL of a 1% (w/v) solution of sodium methoxide in methanol as previously described (Hitz et al., 1994). Following 20 min of incubation at room temperature, fatty acid methyl esters were recovered by the addition of 500 μL of 1 M sodium chloride and extraction with 500 μL of heptane and analyzed using a gas chromatogram (model 5890, Hewlett-Packard, Palo Alto, CA). Fatty acid methyl esters were resolved using an Omegawax 320 column (30-m × 0.32-mm i.d.) (Supelco, Bellefonte, PA), and the oven temperature was programmed from 185°C (3-min hold) to 215°C at a rate of 2.5°C/min. Carrier gas was supplied by a hydrogen generator (Whatman, Clifton, NJ). Fatty acid compositional data presented in Table I were obtained from the analysis of single embryos from the following transformation events: MS185-6-27 (expression of acyl-CoA desaturase), MS190-2-7 (expression of FAE1 homolog), and MS251-2-11 (co-expression of acyl-CoA desaturase and FAE1 homolog).

For the determination of double bond positions, fatty acid methyl esters were converted to dimethyl disulfide derivatives using the method described by Yamamoto et al. (1991). Dimethyl disulfide derivatives were analyzed by GC-MS using a gas chromatograph (model 6890, Hewlett-Packard) interfaced with a mass selective detector (model 5973, Hewlett-Packard). Samples were resolved with a HP-INNOWax column (30-m × 0.25-mm i.d., Hewlett-Packard), and the oven temperature was programmed from 185°C (5-min hold) to 237°C at a rate of 7.5°C/min.

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LITERATURE CITED

Bloomfield DK, Bloch K (1960) The formation of Δ^9-unsaturated fatty acids. J Biol Chem **235:** 337–345

Burg DA, Kleiman R (1991) Preparation of meadowfoam dimer acids and dimer esters and their use as lubricants. J Am Oil Chem Soc **68:** 600–603

Cahoon EB, Carlson TJ, Ripp KG, Schweiger BJ, Cook GA, Hall SE, Kinney AJ (1999) Biosynthetic origin of conjugated double bonds: production of fatty acid components of high-value drying oils in transgenic soybean embryos. Proc Natl Acad Sci USA **96:** 12935-12940

Clemens S, Kunst L (1997) Isolation of a *Brassica napus* cDNA (accession no. AF009563) encoding a 3-ketoacyl-CoA synthase, a condensing enzyme involved in the biosynthesis of very long chain fatty acids in seeds (PGR 97–125). Plant Physiol **115:** 313–314

Dörmann P, Voelker TA, Ohlrogge JB (1995) Cloning and expression in *Escherichia coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long-chain acyl-acyl carrier proteins. Arch Biochem Biophys **316:** 612–618

Doyle JJ, Schuler MA, Godette WD, Zenger V, Beachy RN, Slightom JL (1986) The glycosylated seed storage proteins of *Glycine max* and *Phaseolus vulgaris*: structural homologies of genes and proteins. J Biol Chem **261:** 9228–9238

Erhan SM, Kleiman R, Isbell TA (1993) Estolides from meadowfoam oil fatty acids and other monounsaturated acyl moieties in developing seeds. J Am Oil Chem Soc **70:** 460–465

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryonic suspension culture tissue. In Vitro Cell Dev Biol **27:** 175–182

Fukuchi-Mizutani M, Savin K, Cornish E, Tanaka Y, Ashikari T, Kusumi T, Murata N (1995) Senescence-induced expression of a homologue of Δ9 desaturase in rose petals. Plant Mol Biol **29:** 627–635

Fukuchi-Mizutani M, Tasaka Y, Tanaka Y, Ashikari T, Kusumi T, Murata N (1998) Characterization of Δ9 acyl-lipid desaturase homologues from *Arabidopsis thaliana*. Plant Cell Physiol **39:** 247–253

Hirsinger F (1989) New annual oil crops. *In* G Röbbelen, RK Downey, A Ashri, eds, Oil Crops of the World. McGraw-Hill, New York, pp 518–532

Hitz WD, Carlson TJ, Booth JR Jr, Kinney AJ, Stecca KL, Yadav NS (1994) Cloning of a higher-plant plastid ω-6 fatty acid desaturase cDNA and its expression in a cyanobacterium. Plant Physiol **105:** 635–641

Isbell TA, Abbott TA, Carlson KD (1999) Oxidative stability of vegetable oils in binary mixtures with meadowfoam oil. Ind Crops Prod **9:** 15–123

James DW Jr, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995) Directed tagging of the Arabidopsis FATTY ACID ELONGATION1 (FAE1) gene with the maize transposon activator. Plant Cell **7:** 309–319

Kinney AJ (1996) Development of genetically engineered soybean oils for food applications. J Food Lipids **3:** 273–292

Kunst L, Taylor DC, Underhill EW (1992) Fatty acid elongation in developing seeds of *Arabidopsis thaliana* affecting wax biosynthesis in *Arabidopsis thaliana*. Plant Physiol Biochem **30:** 425–434

Lassner MW, Lardizabal K, Metz JG (1996) A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell **8:** 281–292

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L (1999) CUT1, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a

very-long-chain fatty acid condensing enzyme. Plant Cell **11:** 825–838

Millar AA, Kunst L (1997) Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J **12:** 121–131

Miller RW, Daxenbichler ME, Earle FR, Gentry HS (1964) Search for new industrial oils:VIII. The genus *Limnanthes*. J Amer Oil Chem Soc **41:** 167–169

Moreau RA, Pollard MR, Stumpf PK (1981) Properties of a Δ5 fatty acyl-CoA desaturase in the cotyledons of developing *Limnanthes alba*. Arch Biochem Biophys **209:** 376–384

Phillips BE, Smith CR, Tallent WH (1971) Glycerides of *Limnanthes douglasii* seed oil. Lipids **6:** 93–99

Pollard MR, Stumpf PK (1980). Biosynthesis of C_{20} and C_{22} fatty acids by developing seeds of *Limnanthes alba*: chain elongation and Δ5 desaturation. Plant Physiol **66:** 649–655

Sakamoto T, Wada H, Nishida I, Ohmori M, Murata N (1994) Δ^9 Acyl-lipid desaturases of cyanobacteria: molecular cloning and substrate specificities in terms of fatty acids, *sn*-positions, and polar head groups. J Biol Chem **269:** 25576–25580

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol **49:** 611–641

Strittmatter P, Spatz L, Corcoran D, Rogers MJ, Setlow B, Redline R (1974) Purification and properties of rat liver microsomal stearyl coenzyme A desaturase. Proc Natl Acad Sci USA **71:** 4565–4569

Stukey JE, McDonough VM, Martin CE (1990) The OLE1 gene of *Saccharomyces cerevisiae* encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem **265:** 20144–20149

Taylor DC, Barton DL, Rioux KP, MacKenzie SL, Reed DW, Underhill EW, Pomeroy MK, Weber N (1992) Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore-derived and zygotic embryos of *Brassica napus* L. cv Reston. Plant Physiol **99:** 1609–1618

Thiede MA, Ozols J, Strittmatter P (1986) Construction and sequence of cDNA for rat liver stearyl coenzyme A desaturase. J Biol Chem **261:** 13230–13235

Todd J, Post-Beittenmiller D, Jaworski J (1999) KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J **17:** 119–130

Yamamoto K, Shibahara A, Nakayama T, Kajimoto G (1991) Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts. Chem Phys Lipids **60:** 39–50

Zhang L, Ge L, Parimoo S, Stenn K, Prouty SM (1999) Human stearoyl-CoA desaturase: alternative transcripts generated from a single gene by usage of tandem polyadenylation sites. Biochem J **340:** 255–264